DETECTION OF PATHOGENIC *LEPTOSPIRA* IN WATER AND SOIL FROM SELECTED ENVIRONMENT OF SARAWAK

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Declaration

This Final Year Project is a presentation of my knowledge and own original work except for the acknowledged in text. I hereby declare that no portion of this project has been submitted to any other university or institution of higher learning.

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List of Abbreviations

AGE	-	Agarose gel electrophoresis
bp	-	Base pair
cm	-	Centimeter
°C	-	Degree Celsius
DNA	-	Deoxyribonucleic acid
dNTPs	-	Deoxynucleotide triphosphates
EMJH	-	Ellinghausen-McCullough-Johnson-Harris
EtBr	-	Ethidium bromide
$MgCl_2$	-	Magnesium chloride
mL	-	Milliliter
mM	-	Millimolar
nm	-	Nanometer
ng	-	Nanogram
µL	-	Microliter
µm	-	Micrometer
µM	-	Micromolar
%	-	Percent
PCR	-	Polymerase chain reaction
s	-	Second
TBE	-	Tris-borate-EDTA
UV	-	Ultraviolet
V	-	Volt

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ABSTRACT

The *Leptospira* genus is tightly coiled spirochete from the *Leptospiraceae* family of the *Spirochaetales* order. Based on genotypic classification, 20 *Leptospira* species and over 300 serovars which are categorised into 20 serogroups have been recognized. Pathogenic *Leptospira* which are the causative agents of leptospirosis require hosts for survival and reproduction. Previous studies have reported the presence of pathogenic *Leptospira* from selected environment in Peninsular Malaysia. Since there is limited information about *Leptospira* in environments in Sarawak, therefore, this study is aimed to detect pathogenic *Leptospira* in water and soils from selected environment of Sarawak. A total of 175 water samples and 174 soil samples were collected from Kota Samarahan and Samunsam Wildlife Sanctuary of Sarawak. The samples were cultured in EMJH media supplemented with 5-fluorouracil for 30 days and later subjected to DNA extraction for PCR targeting *lipL32* gene to confirm the pathogenic status of *Leptospira*. The occurrence of pathogenic *Leptospira* is higher in Kota Samarahan with 7.5% (18/240) samples were confirmed to be positive through PCR analysis. Whereas, 4.6% (5/109) samples collected from Samunsam Wildlife Sanctuary exhibited positive leptospiral isolates. In conclusion, this study can provide important information about the occurrence of pathogenic *Leptospira* in environments of Sarawak for epidemiological study of leptospirosis in Malaysia.

Key words: Pathogenic *Leptospira*, EMJH media, 5-fluorouracil, PCR, *lipL32* gene

ABSTRAK

Genus Leptospira adalah Spirochete yang bergelung ketat and berasal dari keluarga Leptospiraceae daripada order Spirochaetales. Berdasarkan klasifikasi genotip, terdapat 20 spesies Leptospira dan lebih daripada 300 serovars yang dikategorikan dalam 20 serogroups telah diiktiraf. Leptospira patogenik yang menyebabkan leptospirosis memerlukan hos untuk terus hidup dan pembiakan. Kajian sebelumnya telah melaporkan kewujudan Leptospira patogenik dalam persekitaran yang terpilih di Semenanjung Malaysia. Oleh sebab maklumat tentang Leptospira di persekitaran Sarawak adalah terhad, kajian ini bertujuan untuk mengesan Leptospira patogenik dalam air dan tanah dari persekitaran terpilih di Sarawak. Sebanyak 175 sampel air and 174 sampel tanah telah dikumpul daripada Kota Samarahan and Samunsam Wildlife Sanctuary di Sarawak. Sampel-sampel tersebut telah dikulturkan dalam media EMJH yang ditambah dengan 5-fluorouracil selama 30 hari. Kemudiannya, DNA diekstrakan daripada sample-sampel tersebut untuk menjalankan PCR menyasarkan lipL32 gen untuk memastikan status patogenik Leptospira. Kewujudan Leptospira patogenik adalah lebih tinggi di Kota Samarahan dengan 7.5% (18/240) sampel telah disahkan sebagai positif melalui analisis PCR. Manakala, 4.6% (5/109) sampel yang dikumpul dari Samunsam Wildlife Sanctuary menunjukkan pencilan Leptospira positif. Kesimpulannya, kajian ini boleh membekalkan maklumat penting tentang kewujudan Leptospira patogenik di persekitaran Sarawak untuk kajian epidemiologi leptospirosis dalam Malaysia.

Kata kunci: *Leptospira* patogenik, media EMJH, 5-fluorouracil, PCR, *lipL32* gen

1.0 Introduction

Leptospirae are tightly coiled spirochaete bacteria which can move with periplasmic flagella at the opposite ends of cell (Levett, 2001). Currently, there are 20 species of *Leptospirae* with over 300 serovars are recognized and the serovars are categorized into 20 serogroups (Mayer-Scholl *et al.*, 2014). Saprophytic *Leptospirae* can be found freely in environment and play no role in human infection because these organisms are not necessarily resided in host and they feed on organic matter in water (Lim *et al.*, 2011). Whereas, pathogenic *Leptospirae* are the causative agents of leptospirosis which is a zoonotic disease that affects animals as well as humans. These bacteria require hosts for survival and reproduction and they are particularly discovered in renal tubules of maintenance hosts (Lim *et al.*, 2011).

Leptospirae are obligate aerobes that grow optimally at temperatures from 28 to 30 °C (Levett, 2001). These bacteria can also exist in the natural environment such as water and soil after they are shed into the urine of infected hosts (Rawlins *et al.*, 2014). The survival of pathogenic *Leptospirae* is greatly dependent on the ambient temperature, moisture and pH values of soil (Ridzlan *et al.*, 2010). *Leptospirae* can survive in the environment with high humidity, but cannot withstand drought or hypertonicity and temperatures higher than 50 °C (Levett, 2001; Mohammed *et al.*, 2011). These spirochetes can also survive in alkaline condition with the pH value up to 7.8 (Mohammed *et al.*, 2011).

As compared to saprophytic *Leptospirae* which are the non-disease causing bacteria, pathogenic *Leptospirae* do not show better survival in the external environment because these bacteria are less resistant to ultraviolet radiation, extreme temperature, osmotic pressure, pH, and humidity (Ananyina, 2010). However, if the environmental

conditions meet the survival requirements of these spirochetes, *Leptospirae* may thrive and survive for longer period of time (Ridzlan *et al.*, 2010). This can pose a risk of leptospirosis when human are exposed to the environment contaminated with *Leptospirae*.

Leptospirosis is usually found in tropical or subtropical countries including Malaysia due to better survival of pathogenic *Leptospirae* in warm and humid environment (Zavitsanou & Babatsikou, 2008). *Leptospirae* also cause water-borne diseases as the infection reported is usually linked to water-related activities. Leptospiral infection among athletes who participated in the Eco-Challenge-Sabah 2000 which involved water-related activities has been reported (Lim *et al.*, 2011). An outbreak of leptospirosis that involved three fatalities has been found to be related to a public recreational lake in Hutan Lipur Yu, Maran, Malaysia (Benacer *et al.*, 2013). Another outbreak has been reported at RSAT Army camp in Kuching, Sarawak with five army recruits were infected with leptospirosis due to water activities in the river close to the camp (Thayaparan *et al.*, 2013). An increasing number of leptospirosis cases in Sarawak were reported from 49 cases in year 2010 to 597 cases in the year 2014 (Thayaparan *et al.*, 2013; Sarawak Health Department, 2015). Thus, leptospirosis infection is a great concern in Sarawak.

The understanding of dynamic changes in the epidemiology of leptospirosis is not just about the isolation of *Leptospira* from the humans and animals, but the environmental water and soil as well (Saito *et al.*, 2013). Previous studies have reported the presence of pathogenic *Leptospira* from selected environment (water and soils) in Peninsular Malaysia (Ridzlan *et al.*, 2010; Benacer *et al.*, 2013). There were 29 pathogenic serovars had been isolated by Alexander *et al.* (1975) from natural water and wet soils in Malaysia. *Leptospira* serovar *Hebdomadis* was detected in environmental samples collected from Terengganu (Ridzlan *et al.*, 2010). However, there is not much information about

leptospirosis in the environment of Sarawak and the epidemiological study of leptospirosis in Sarawak could be impeded. Therefore, this study was conducted to detect the pathogenic *Leptospira* in water and soils from selected environments of Sarawak.

The objectives in this study were:

1. To isolate pathogenic *Leptospira* from water and soils samples in environments of Sarawak using Ellinghausen-McCullough-Johnson-Harris (EMJH) medium with additional 5-fluorouracil.
2. To determine the occurrence of pathogenic *Leptospira* in water and soil samples from selected environments of Sarawak by detecting the presence of *lipL32* gene in pathogenic *Leptospira* using polymerase chain reaction (PCR) technique.

2.0 Literature Review

2.1 Classification of *Leptospira*

The *Leptospira* genus is tightly coiled spirochete from the *Leptospiraceae* family of the *Spirochaetales* order. The *Leptospira* genus is serologically classified into two species which are *Leptospira interrogans* containing all pathogenic strains and *Leptospira biflexia* comprising all saprophytic strains (Evangelista & Coburn, 2010). The abilities of *L. biflexa* to grow in the presence of 8-azaguanine and at the temperature of 13 °C enable it to be differentiated from *L. interrogans* (Levett, 2001). However, *L. biflexa* cannot form cells in spherical shape in 1M sodium chloride (NaCl) (Levett, 2001). The genotypic classification which is based on DNA hybridization studies or 16S rRNA gene sequencing has defined 20 species within *Leptospira* genus and the species are clustered into saprophytic, pathogenic, and intermediate *Leptospira* (Levett, 2001; Levett & Haake, 2009; Mayer-Scholl *et al.*, 2014). The genomic species are defined based on genetic homology in which DNAs have 5% or less unpaired bases and show homology of above 60% or 70% when the reassociation temperatures are 70 °C and 55 °C respectively (Vijayachari *et al.*, 2008). *Leptospira* species through genotypic classification is displayed in Table 1.

The expression of epitopes in a mosaic of the lipopolysaccharides (LPS) antigens is used to classify *Leptospira* into serovars and the antigenically related serovars can form into serogroups (Levett & Haake, 2009; Mohammed *et al.*, 2011). The serovars can be defined by agglutination test through cross-absorption with homologous antigen. To date, over 250 pathogenic serovars have been recognised and categorised into 25 serogroups of *Leptospira* genus (Ahmed *et al.*, 2012).

Table 1: *Leptospira* species through genotypic classification (Ahmed *et al.*, 2012).

Pathogenicity	*Leptospira* species
Pathogenic *Leptospira*	*L. interrogans*, *L. santarosai*, *L. weilli*, *L. borgpetersenii*, *L. noguchii*, *L. kirschneri*, *L. alexanderi*, *L. alstonii*, *L. kmetyi*
Non-pathogenic / Saprophytic *Leptospira*	*L. biflexa*, *L. wolbachii*, *L. vanthielii*, *L. terpstrae*, *L. yanagawae*, *L. meyeri*
Intermediate *Leptospira*	*L. inadai*, *L. fainei*, *L. broomii*, *L. wolffii*, *L. licerasiae*

2.2 Morphology of *Leptospira*

Leptospirae have dimensions of approximately 0.1 μm in width by 6-20 μm in length (Levett, 2001). Either one or both of its pointed ends are bent into distinctive hooks and hence it is given a shape of question-mark (Levett, 2001; Evangelista & Coburn, 2010). Motility of *Leptospirae* is aided by two periplasmic flagella which are located within the periplasmic space (Levett, 2001). This bacterium shows characteristics of Gram-negative and Gram-positive bacteria because it has a double membrane structure and lipopolysaccharides (LPS) on the outer surface as well as close association between peptidoglycan cell wall and cytoplasmic membrane (Haake, 2000). However, it has a lower endotoxic activity if compared to Gram-negative bacteria (Levett, 2001). Since their sizes are small, phase contrast or dark field microscopy is required to observe these bacteria (Levett & Haake, 2009).

2.3 Epidemiology of Leptospirosis

Leptospirosis is an endemic disease in Southeast Asia, Latin America, Oceania, Caribbean, and the Indian subcontinent (Evangelista & Coburn, 2010). The annual incidence rate is estimated from 0.1 to 1 per 100,000 populations in temperate regions and from 10 to 100 per 100,000 populations in tropical regions (Verma *et al.*, 2013). Leptospirosis is recognized as the important emerging worldwide disease in Southeast Asia as its warm and humid climatic condition with high precipitation rate has met the survival requirements of *Leptospira* (El Jalii & Bahaman, 2004; Khairani-Bejo *et al.*, 2004). Hence, *Leptospira* have been isolated from water and soils in Malaysia (Alexander *et al.*, 1975; Ridzlan *et al.*, 2010). In Malaysia, the estimated incidence rate of leptospirosis ranges from 2 to 5 per 100,000 populations, but it has shown a significant increase in recent years (Lim *et al.*, 2011). According to the Ministry of Health Malaysia (2011), the number of case of leptospirosis had increased from 263 with 20 deaths in the year 2004 to 1418 with 62 deaths in the year 2009.

2.4 Clinical Manifestation

The clinical manifestation of leptospirosis can be divided into two forms in which 90% of patients present anicteric symptoms and 10% of them suffer from icteric syndromes (Dutta & Christopher, 2005). The anicteric and icteric leptospirosis can be presented in two phases, which are septicemic phase and immune phase (Gamage *et al.*, 2011). In septicemic phase, patients can have febrile illness for about a week and *Leptospira* can be recovered from cerebrospinal fluid (CSF) and blood, whereas the immune phase involves the production of antibody and *Leptospira* can be detected in the tissues, organs, and urine of patients (Levett & Haake, 2009; Gamage *et al.*, 2011). For some patients, leptospirosis can present the two distinct phases with initial septicemic phage followed by temporary decline in fever and it is followed by immune phase (Levett & Haake, 2009). However, most of the patients show symptoms which are started at second phase of illness (Levett & Haake, 2009). Anicteric leptospirosis is mild form of disease which is presented as influenza-like symptoms such as severe myalgias, vomiting, sudden high fever, and headache. Icteric leptospirosis is severe form of disease which is associated with jaundice, renal impairment and haemorrhage (Dutta & Christopher, 2005). Patients who develop severe disease leptospirosis will have mortality rates of approximately 5% to 40% (Agudelo-Flórez *et al.*, 2009).

2.5 Transmission of Leptospirosis

The leptospirosis transmission involves the interaction between reservoir animals, existence of *Leptospira* in environment and humans (Lau *et al.*, 2010). Transmission of *Leptospira* can be occurred through direct contact with urine, body fluids or tissues of reservoir animals primarily rodents or indirectly contact with urine contaminated water and soils (Ngbede *et al.*, 2012; Rawlins *et al.*, 2014). *Leptospira* can be transmitted through transplacental, breast-feeding by infected mother or sexual contact (Vijayachari *et al.*, 2008). Consumption of contaminated drink and food also can cause leptospirosis (Verma *et al.*, 2013). *Leptospira* can enter and infect human body through abrasions or cuts in the skin or through the mucous membrane of mouth, nose, and eyes (Levett, 2001; Lim *et al.*, 2011). Prolonged immersion in water poses a risk of *Leptospira* infection through intact skin, particularly with abrasions (Levett, 2001). Therefore, exposure to contaminated environment poses high risk of transmission to humans and animals.

A communal relationship has been found between some vertebrate animal species and *Leptospira* in which these animals become natural maintenance hosts for pathogenic *Leptospirae* that reside in their kidneys (Vinodkumar *et al.*, 2011). These maintenance hosts usually manifest mild or no symptoms after infection, but these bacteria would be excreted in their urine for a long period of time and normally in increasing amounts (Cosson *et al.*, 2014; Mayer-Scholl *et al.*, 2014). However, humans are known as incidental hosts and could be suffered from severe and fatal infectious (Evangelista & Coburn, 2010).

2.6 Laboratory Diagnosis

2.6.1 Isolation

Isolation of *Leptospira* can be performed by using culturing method which requires specific media. *Leptospira* can grow in media supplemented with vitamins, ammonium salts and long-chain fatty acids which act as the sole carbon source for *Leptospira* through metabolism with β-oxidation (Levett, 2001). According to Levett (2001), Ellinghausen-McCullough-Johnson-Harris (EMJH) medium is most commonly used medium for culturing *Leptospirae* in the laboratory. However, the non-selective EMJH medium is not able to recover high percentage of *Leptospirae* (Miraglia *et al.*, 2009). Hence, a selective agent called 5-fluorouracil is added in culture media to minimize the growth of contaminants (Ridzlan *et al.*, 2010).

5-fluorouracil is a pyrimidine analogue and its strong bacteriostatic action could inhibit the growth of many bacteria (Johnson & Rogers, 1964). Since fluorouracil may not enter the cells and not used up by *Leptospira*, the pyrimidine analogue can be used for selectively isolate *Leptospira* from the contaminants (Johnson & Rogers, 1964). It has been reported that the recommended concentrations of 5-fluorouracil for selective isolation of *Leptospirae* are between 100 μg/mL and 400 μg/mL (Oie *et al.*, 1986). Higher concentrations of this chemical could not be used because the growth of pathogenic *Leptospira* might be inhibited (Tansuphasiri *et al.*, 2006). 5-fluorouracil with concentration of 100 μg/mL was used in the previous study as all serovar strains of *Leptospirae* can grow at this concentration (Oie *et al.*, 1986). EMJH medium supplemented with 5-flurouracil has been used in the previous studies for isolation of *Leptospira* from environmental water and soil (Issazadeh *et al.*, 2009; Yuwvaranni & Thiruvengadam, 2010; Benacer *at al.*, 2013). The growth of *Leptospira* is slow and usually takes several weeks (Levett & Haake, 2009).

2.6.2 Molecular Diagnosis and Typing

2.6.2.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technique that can generate millions or more copies of a particular DNA sequence by amplifying even a single copy of DNA. Development of PCR contributes to gene analysis, diagnosis of genetic diseases as well as detection of pathogens such as bacteria, virus, and fungi (Valones *et al.*, 2009). This technique has been used in detection of *Leptospira* environmental samples such as animals, water, and soil (Agudelo-Flórez *et al.*, 2009; Rawlins *et al.*, 2014). PCR performs better than microscopic agglutination test (MAT) in detection of *Leptospira* especially during the early stage of leptospirosis where immunoglobulin M (IgM) antibodies have not been appeared in serum (Levett & Haake, 2009). Specific *Leptospira* species can be detected by PCR through using specific primers for amplifying target genes (Gravekamp *et al.*, 1993; Mérien *et al.*, 1992; Murgia *et al.*, 1997).

Pathogenic *Leptospira* can be detected by designing specific primers which target on *lipL32* gene (Vein *et al.*, 2012). The *lipL32* gene encodes LipL32 lipoprotein which is the major outer membrane protein of *Leptospira* and this lipoprotein has made up over 75% of the total proteins weight (Hoke *et al.*, 2008; Kucerova *et al.*, 2013). This gene is highly conservative in the pathogenic *Leptospirae* in which the conformity of primary sequence of amino acids can be up to 98% (Kucerova *et al.*, 2013). Since *lipL32* gene is highly conservative and only present in pathogenic *Leptospira* but not in saprophytic ones, detection of this gene is able to confirm the pathogenic status of *Leptospira* from the samples (Kucerova *et al.*, 2013). From the previous studies, specific-PCR that targets *lipL32* gene has been successfully detected pathogenic *Leptospira* from the environmental samples (water and soil) (Ghane & Yasouri, 2013; Rawlins *et al.*, 2014).

3.0 Materials and Methods

The lists of materials and apparatus used in this study are stated in Appendix 1.

3.1 Study Sites

In the present study, Kota Samarahan areas and Samunsam Wildlife Sanctuary were chosen as the sampling sites for water and soil samples collection. The locations of these sampling sites were shown in Figures 1 and 2. These two sites respectively represent places with relatively high and no human populations. A total of 240 water (n=120) and soil (n=120) samples were collected from 4 selected sites (Kampung Sungai Mata, Desa Ilmu, Kampung Plaie, and Kampung Sebayor) within Kota Samarahan. The water and soil samples were collected randomly from lake, landfills, drain waters, river, villages, and paddy fields. The water and soil samples had also been collected from Samunsam Wildlife Santuary which is the oldest wildlife sanctuary in Malaysia located in the Kuching division. A total of 55 water and 54 soil samples had been collected at different locations within the wildlife sanctuary. The water samples were mainly collected from the main river of Samunsam Wildlife Santuary, stagnant water, and swamp water in the forest and pipe water which is from the dam at the upstream river. The soil samples were collected from the forests and areas of rest houses. The details of water and soil samples collected from the sampling sites were shown in Table 2.

Table 2: Details of water and soil samples which were investigated with proposed methodology

Sampling site	Coordinates	Number of water samples	Number of soil samples
Kota Samarahan			
1. **Kampung Sebayor**	N1°27'53.514" E110°26'33.0108"		
Source of water samples			
• Drain water		10	
• Stagnant water		6	
• River water		14	
Source of soil samples			
• Surroundings of houses			30
Subtotal		**30**	**30**
2. **Kampung Plaie**	N1°27'40" E110°25'52"		
Source of water samples			
• Drain water (in paddy fields)		17	
• Stagnant water (in paddy field)		1	
• Stream water		9	
• Stagnant water (near landfill)		3	
Source of soil samples			
• Paddy field			15
• Landfill			15
Subtotal		**30**	**30**
3. **Kampung Sungai Mata**			
Source of water samples			
• Stagnant water (in paddy field)	N1°26'30.075" E110°32'4.473"	15	
• Stream water	N1°26'30.075" E110°32'4.473"	5	
• Drain water	N1°26'42.9432" E110°31'12.6192"	10	
Source of soil samples			
• Paddy field	N1°26'30.075" E110°32'4.473"		15
• Barn	N1°27'46.3608" E110°31'3.9252"		5
• Surroundings of houses	N1°26'42.9432" E110°31'12.6192"		10
Subtotal		**30**	**30**
4. **Desa Ilmu**			
Source of water samples			
• Drain effluent water	N1°27'13.7844" E110°27'27.7164"	10	
	N1°27'18.7848" E110°27'1.1664"	6	
	N1°27'19.8" E110°26'57.462"	4	
• Lake water	N1°26'50.6256" E110°27'35.5968"	10	
Source of soil samples			
• Landfills	N1°27'13.7844" E110°27'27.7164"		10
	N1°27'18.7848" E110°27'1.1664"		5
	N1°27'19.8" E110°26'57.462"		5
• Surroundings of lake	N1°26'50.6256" E110°27'35.5968"		10
Subtotal		**30**	**30**
Samunsam Wildlife Sanctuary	Coordinate was not taken		
Source of water samples			
• Swamp water (in tropical forest)		12	
• River water (Samunsam River)		36	
• Stagnant water (in tropical forest)		3	
• Pipe water		4	
Source of soil samples			
• Tropical forest			39
• Riverine forest			9
• Surroundings of rest houses			6
Subtotal		**55**	**54**
Total		**175**	**174**

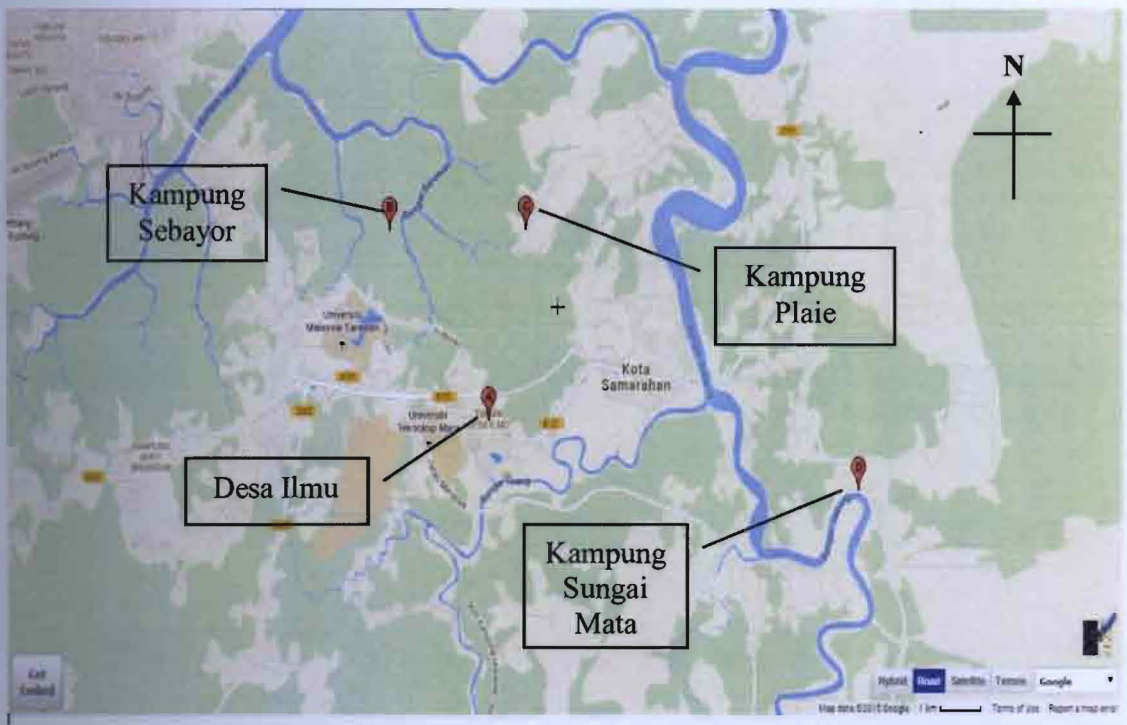

Figure 1: Map showing the sampling sites within Kota Samarahan areas in this study. A represents Desa Ilmu; B represents Kampung Sebayor; C represents Kampung Plaie; D represents Kampung Sungai Mata. (Map produced using Scribble Maps, https://www.scribblemaps.com/create/)

Figure 2: Map showing the sampling site (Samunsam Wildlife Sanctuary) in this study (Wood, 2007).

3.2 Sample Collection

Water and soil samples were collected from Samunsam Wildlife Sanctuary and Kota Samarahan areas for the detection of pathogenic *Leptospira*. Fifty millilitres of water samples were collected using sterile 50 mL Falcon tube. Topsoil of approximately 20 g was collected using spatula and placed in sterile 50 mL Falcon tube. The temperatures and pH values of water and soils were measured at the site by using thermometer and PH100-ExStik pH meter (Extech, USA) respectively.

3.3 Sample Processing and *Leptospira* Isolation

All the samples collected were processed as described by Benacer and colleagues (2013) with some modifications. The pH values of water and soil were measured again using PH100-ExStik pH meter (Extech, USA) during sample processing in laboratory. Fifty millilitres of water sample was filtered using Minisart NML syringe filter (Sartorious AG, Germany) with 0.20 µm pore size. One millilitre of filtered water was inoculated into modified liquid EMJH medium which contained 5-fluorouracil to minimize the growth of contaminants. Soil sample (10 g) was soaked in distilled water which was about three times the volume of samples. The sample was shaken vigorously and was settled for 15 to 20 minutes. The filtered water (1 mL) was inoculated into EMJH culture medium. The inoculated media were incubated aerobically at the room temperature for 30 days.

3.4 Genomic DNA Extraction

Genomic DNA was extracted from 30-day-old fresh culture using Wizard Genomic DNA Purification Kit (Promega, Wisconsin, USA) according to manufacturer's instructions. Briefly, 1.5 mL of bacterial culture was pipetted into 1.5 mL microcentrifuge tube and the sample was then centrifuged at 10700 rpm for 5 minutes. The supernatant was discarded and 600 μL of Nucleic Lysis Solution was added to the pellet. After the mixture was mixed vigorously by vortexing, it was then incubated at 80 °C for 5 minutes. Three microlitres of RNase solution was added to the cell lysate. The mixture was incubated at 37 °C for 30 minutes and later chilled on ice for 5 minutes. Two hundred microlitres of Protein Precipitation Solution was added before incubation on ice for another 5 minutes. After centrifugation at 10700 rpm for 3 minutes, 600 μL of supernatant was transferred to a new microcentrifuge tube which containing 600 μL of isopropanol. The mixture was centrifuged at 10700 rpm for 2 minutes and the supernatant was discarded. Six hundred microlitres of 70% ethanol was added into the tube and centrifuged at 10700 rpm for another 2 minutes. The tube was drained on a clean absorbent paper and then air dried for 15 minutes. After that, 100 μL of DNA rehydration solution was added to the tube. The DNA template was used in the subsequent experiment.